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Digestive Proteinases of Alfalfa Weevil, *Hypera postica*, (Gyllenhal) (Coleoptera: Curculionidae)

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The alfalfa weevil, *Hypera postica* (Gyllenhal), is a serious economic pest of alfalfa in the State of Kansas. To identify potential insecticidal proteins that may be incorporated into transgenic alfalfa to enhance resistance to the weevil, the digestive proteinases of larval stages of *H. postica* were characterized. Seven caseinolytic proteinase activities from both serine and cysteine subclasses were identified. Five of the activities tentatively were stimulated by thiol-reducing reagents and were identified as cysteine proteinases. Substrate and inhibitor analysis identified the other two activities as chymotrypsin-like enzymes. These results suggest that genes encoding inhibitors of cysteine proteinases and serine proteinases, especially those that inhibit chymotrypsin-like enzymes, could be used to transform alfalfa and reduce damage by weevils.

INTRODUCTION

The alfalfa weevil, *Hypera postica* (Gyllenhal), is a coleopteran pest inflicting serious economic damage to alfalfa, *Medicago sativa* L. The weevil occurs in most cultivated alfalfa in the United States and has been a problem in alfalfa grown in Kansas for many years (Higgins, Blodgett, and Lenssen, 1988, 1989). Although both adult and larval stages feed on alfalfa, the major damage is inflicted by larvae. The weevil normally completes one generation each year, although after mild winters in Kansas, infestations begin earlier and may promote the occurrence of a second generation (Higgins, Blodgett, and Lenssen, 1988). Biological control, including the use of parasitic insects and entomopathogenic fungi, has significantly reduced insecticide treatments for control of the alfalfa weevil in the northeastern United States (Day, 1981). However, other regions have had less success with biological control and continue to use insecticides to reduce weevil damage. Traditional breed-

ing of alfalfa for resistance to the alfalfa weevil has been largely unsuccessful.

Alfalfa weevil larval feeding removes foliar tissues from alfalfa plants, reducing the protein content and digestibility of the harvested alfalfa hay. Dry matter losses have exceeded 6 tons per hectare in the first cutting in some years (Higgins, Blodgett, and Lenssen, 1989). Severe damage to the first cutting may result in indirect residual losses of an additional 4.5 tons per hectare through delayed growth and reduced production in later cuttings (Higgins, Blodgett, and Lenssen, 1988).

Modification of economically important crops by the addition of insecticidal proteins is a potentially effective method to control pests. Genes encoding digestive proteinase inhibitors have been incorporated into plants with differing results (reviewed in Oppert, 2000). One factor that has limited the effectiveness of insect control with transgenic plants containing proteinase inhibitors has been compensation by insect pests for the presence of the exogenous inhibitor, either by increasing the production of the targeted proteinase or by synthesis of inhibitor-insensitive enzymes (Oppert, 2000). This compensation results in poor efficacy of the transgenic plant in reducing pest damage.

The most effective design of inhibitor-transgenic crops encompasses a three phase approach. Initially, information is needed on the digestive proteinase subclasses in the target pest. Four mechanistic classes of proteinases are recognized by the International Union of Biochemistry and include serine, cysteine, aspartic, and metallo-proteinases (Neurath, 1993). The mechanistic class of a proteinase can be predicted by the use of diagnostic substrates, inhibitors, and activators, or by DNA or protein sequence comparison. Once specific proteinase-types are identified, artificial diet bioassays of inhibitors identify candidate insecticidal inhibitors. Finally, incorporation of the gene or genes encoding proteinase inhibitors into the plant genome permits evaluation of the efficacy of the inhibitor-expressing transgenic plant.

In bioassays, Elden (1995) determined that inhibitors of cysteine proteinases at 0.1% (w/w) reduced the feeding, growth, and development of alfalfa weevil larvae. The contents of the larval midgut had a pH of 6.6 (Elden, 1995), which is in the range of activity for insect cysteine proteinases (Reeck and others, 1999). Therefore, the major proteinases of alfalfa weevil digestion were predicted to be those from the cysteine subclass. Leupeptin, an inhibitor of proteinases from both serine and cysteine proteinase subclasses, decreased larval growth and survival for nine generations (Elden, 2000). However, soybean Bowman-Birk trypsin-chymotrypsin inhibitor at 1.0% had an effect similar to cysteine proteinase inhibitors, suggesting that serine proteinases also may aid in alfalfa weevil larval digestion (Elden, 1995).

Both serine and cysteine proteinase subclasses have been demonstrated in other insects in the family Curculionidae. Trypsin-like activities occur in the

granary weevil, *Sitophilus granarius* (Linnaeus), and the sweetpotato weevil, *Cylas formicarius elegantulus* (Summers) (Baker, 1982; Baker, Woo, and Muller, 1984). Grain dusted with high levels of soybean trypsin inhibitor was protected from damage by rice weevils, *S. oryzae* (Linnaeus) (Su, Speirs, and Mahany, 1974). In the maize weevil, *S. zeamais* Motschulsky, a thiol-activated cysteine proteinase and a chymotrypsin-like proteinase were identified (Houseman and Thie, 1993). Transgenic strawberry expressing a trypsin inhibitor transferred from cowpea had reduced damage by the vine weevil, *Otiorhynchus sulcatus* (Fabricius) (Graham, Gordon, and McNicol, 1997). Digestive proteinases of the cabbage seed weevil, *Ceutorhynchus assimilis* (Paykull), were inhibited *in vitro* by the cysteine proteinase inhibitor oryzacystatin I (OCI) (Girard and others, 1998). However, successful expression of OCI in transgenic oilseed rape did not affect weevil survival (Girard and others, 1998).

There has been reduced damage in transgenic alfalfa plants incorporating insecticidal proteins with activity against insect pests. A gene encoding a δ -endotoxin from the bacterium *Bacillus thuringiensis* was transferred to alfalfa and enhanced the resistance of alfalfa to armyworm *Spodoptera* sp. (Strizhov and others, 1996). Proteinase inhibitors also have been transferred into alfalfa. A wound-inducible serine proteinase inhibitor from tomato was incorporated into transgenic alfalfa, but no data were reported on the efficacy of protection from alfalfa pests (Narvárez-Vásquez, Orozco-Cárdenas, and Ryan, 1992). A gene encoding an anti-elastase inhibitor from the tobacco hornworm, *Manduca sexta* (Linnaeus), was transferred to alfalfa, resulting in a delayed feeding by thrips (Thomas and others, 1994).

As a first step to develop alfalfa plants with enhanced resistance to the alfalfa weevil, we have characterized the digestive proteinases of field-collected alfalfa weevils. We have used specific substrates, inhibitors, and activators to detect major serine and cysteine subclasses of digestive proteinases of the alfalfa weevil larvae.

MATERIALS AND METHODS

Insect dissection. Larvae were collected from an alfalfa field in Riley County, Kansas, and brought to the laboratory on alfalfa leaves for immediate dissection. Fourth instar larvae weighed an average of $11.8 \text{ mg} \pm 0.9$ and had an average head capsule size of $0.63 \text{ mm} \pm 0.01$ ($n = 40$). Prior to dissection larvae were chilled, and the posterior and anterior ends were removed. Guts were excised, immediately submersed in $25 \text{ }\mu\text{l}$ of ice-cold buffer A (200 mM Tris, pH 8.0, 20 mM CaCl_2) per gut, and frozen at -20°C . For assays, samples were thawed, vortexed briefly, and centrifuged at $15,000 \times g$ for 5 min, and the supernatant containing soluble proteins was used.

Microplate Proteinase Assays. The procedure was adapted from a previously described microplate assay (Oppert, Kramer, and McGaughey,

1997). For pH curves, 0.40 gut equivalent was diluted into 90 μ l of buffers of various pH, obtained using a universal buffering system (Frugoni, 1957). The substrate, 10 μ l containing 0.1 μ g fluorescently labeled casein (BOD-IPY-TR-X casein, Molecular Probes, Eugene, OR), was added to each well to initiate the reaction. Samples were incubated at 37°C, and the fluorescence was measured (excitation 584; emission 620) and corrected by subtracting readings obtained with incubations of substrate only (no enzyme). Measurements of enzyme and buffer or buffer only produced negligible fluorescence.

Where indicated, reducing conditions were achieved by adding L-cysteine, dithiothreitol (DTT), and β -mercaptoethanol (ME) to buffers at 1 or 5 mM concentrations. Proteinase inhibitors used in this study included 1.0 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), 1.0 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM chymostatin, all obtained from Sigma Chemical Company (St. Louis, MO). Inhibitors were added to the indicated buffer \pm 5 mM L-cysteine and preincubated with gut extract for 30 min before the addition of substrate. All incubations were at 37°C.

To evaluate serine proteinase activities, class-specific substrates conjugated to p -nitroanilide were obtained from Sigma and included: N- α -benzoyl-L-arginine p -nitroanilide (BAPNA), specific for trypsin-like proteinases; N-succinyl ala-ala-pro-phe p -nitroanilide (SAAPFpNA), specific for chymotrypsin-like proteinases; and leucine p -nitroanilide (LpNA), specific for leucine aminopeptidase-like exopeptidases. Substrates were diluted in 200 mM Tris-HCl, pH 8.0, 50 mM CaCl₂ (Buffer B). This buffer previously has demonstrated good solubility for p -nitroanilide substrates with low auto-hydrolysis. To initiate the reaction, 50 μ l of 0.08 gut equivalents of alfalfa weevil larval gut proteinases diluted in Buffer B were added to each well. Samples were incubated at 37°C for 5 min, and absorbance was read at 405 nm in 15 sec intervals. The change in absorbance per min was calculated by the software KinetiCalc3 (BIO-TEK, Winooski, VT) and the data were converted to μ mol per min per mg of protein in each gut extract.

Zymogram Analyses. Aliquots of gut proteins (0.6 gut equivalents per well) were separated by nonreducing sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) using 4–12% ZBC gels (containing casein) and MultiMark molecular mass markers (NOVEX, San Diego, CA). Gels were incubated for 30 min in 2% triton-X 100, rinsed in deionized water, cut into strips, and developed in universal buffer (Frugoni, 1957) at pH 4, 7, or 10, with and without 5 mM L-cysteine for 4 h. Because the gels contained Commassie-stained casein, the appearance of clear zones indicating active proteinases was monitored during the developing period.

Proteinase Activity Blots. Gut extracts (5 gut equivalents per lane) from alfalfa weevil larvae were subjected to SDS-PAGE using 10–20% tricine gels (NOVEX). Gels were incubated in 2% triton-X 100 for 30 min, rinsed

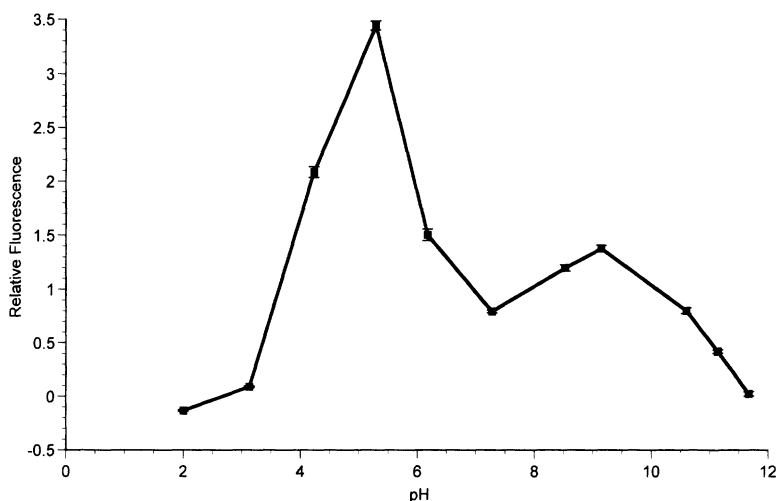


Figure 1. Caseinolytic activity of alfalfa weevil gut extract at different pH values. Fluorescence was read after 1 h incubation and corrected by subtracting values obtained with substrate only from those with gut extract + substrate ($n = 3$).

in deionized water, electrotransferred to nitrocellulose, and incubated with p -nitroanilide substrates in buffer A and developed as previously described (Oppert and Kramer, 1998).

Statistical Analyses. Data were analyzed with the Tukey test comparison and were reported as mean \pm standard error (ANOVA) (Statmost, 1994).

RESULTS AND DISCUSSION

Enzymes that may be involved in protein digestion were extracted from the digestive tract of alfalfa weevil larvae and evaluated for caseinolytic activity at different pH values (Fig. 1). After fluorescence was corrected for background values, a major peak of activity was observed around pH 5, with a smaller, broader peak around pH 9. Insect proteinase activities at lower pH values, such as those in the larger peak in Figure 1, may be associated with cysteine proteinases, whereas activities at higher pH values usually are the result of serine proteinase activities (Reeck and others, 1999).

The effects of thiol activators on the caseinolytic activity of alfalfa weevil proteinases were evaluated at pH 4, 7, and 10 (Fig. 2). At pH 4, all activators significantly increased the caseinolytic activity of gut extracts, with L-cysteine resulting in the largest increase in activity. At pH 7, a significant but less pronounced increase in activity was observed with activators, with largest increases observed when DTT and β -mercaptoethanol were added. A small increase in activity was observed at pH 10 with all of the activators. Increases in activity with thiol-reducing reagents at lower pH values usually

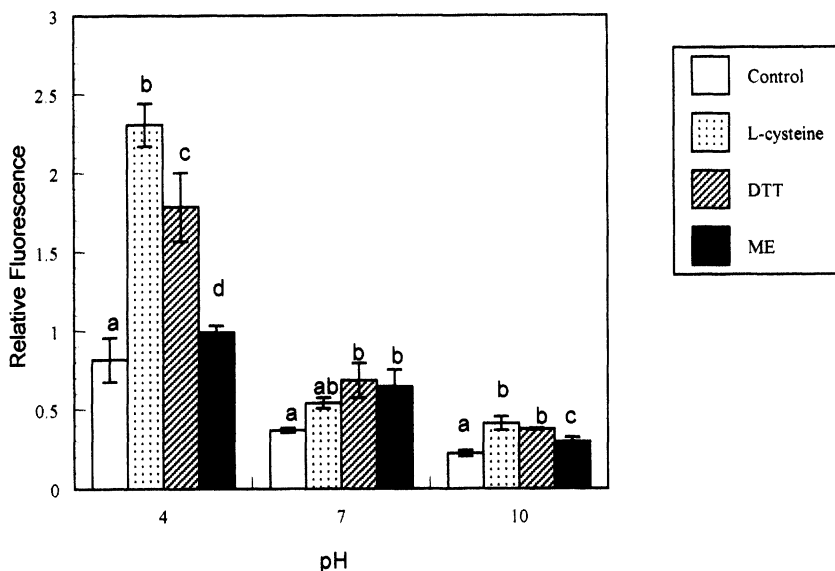


Figure 2. Effect of thiol reducing reagents on caseinolytic activity of alfalfa weevil gut extract at different pH values. Values were obtained following 1 h incubation. Mean \pm S.E. ($n = 3$). Mean fluorescence values with same letter are not significantly different ($p < 0.05$) within each pH group.

are associated with cysteine proteinases, suggesting the presence of cysteine proteinases in alfalfa weevil larvae.

The effects of the proteinase inhibitors chymostatin, E-64, leupeptin, and PMSF were tested with the alfalfa weevil gut extract at pH 4 and 9 (Fig. 3). Chymostatin inhibits chymotrypsin-like enzymes, E-64 inhibits cysteine proteinases, leupeptin inhibits cysteine and serine proteinases, and PMSF inhibits serine and some cysteine proteinases (Roche Molecular Biochemicals, 2000). E-64 also can inhibit some insect proteinases (Reeck and others, 1999). PMSF was not an effective inhibitor of caseinolytic activity in any of the conditions tested, whereas chymostatin was effective in all conditions (Fig. 3). Chymostatin, E-64, and leupeptin were effective inhibitors at pH 4 under reducing conditions (Fig. 3C). Significantly higher inhibition was observed with chymostatin under nonreducing conditions at pH 9 and reducing conditions at pH 4, and this may be indicative of both chymotrypsin-like and cysteine proteinase activities. Inhibition by E-64 and leupeptin at pH 4 only under reducing conditions suggests the presence of active cysteine proteinases at these conditions. Combined, we interpret from the data the presence of serine and cysteine proteinase activities in lower pH buffers, while chymotrypsin-like serine proteinases are active at higher pH (Figs. 1–3).

Zymogram analysis was used to examine further the caseinolytic activity

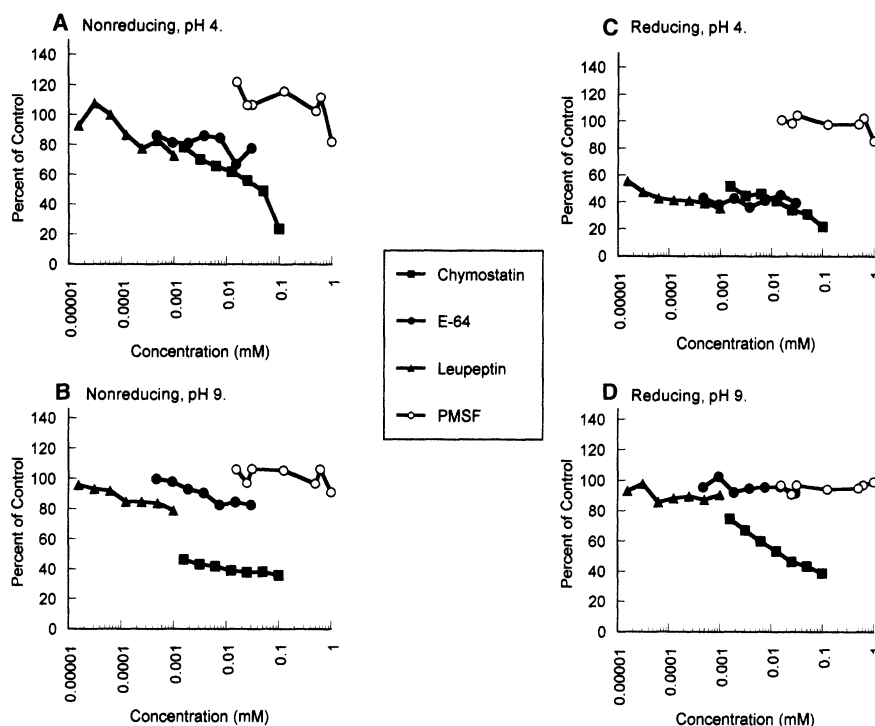


Figure 3. Effect of proteinase inhibitors on caseinolytic activity of alfalfa weevil gut extract at pH 4 (A, B) and pH 10 (C, D) under reducing (A, C) and nonreducing (B, D). Following a 30 min preincubation of inhibitor and enzyme, substrate was added and read after 6.5 h. Mean \pm S.E. (n = 3).

of the alfalfa weevil gut extract (Fig. 4). In buffer containing L-cysteine, seven distinct proteinases were identified. Five proteinases were active when the gel was incubated in pH 4 buffer (P2, P4, P5, P6, P7), five were active in pH 7 buffer (P2, P3, P4, P5, P6), and two were active in pH 10 buffer (P1 and P3). In the absence of L-cysteine, two proteinase activities were observed at pH 4 (P2 and P4), pH 7 (P3 and P4), and pH 10 (P1 and P3). P2, P4, P5, P6, and P7 activities were greater in the presence of L-cysteine, suggesting that these enzymes were thiol-activated proteinases. P1 and P3 activities, unaffected by L-cysteine in the buffer, were abolished when the incubation buffer contained chymostatin, suggesting that these were chymotrypsin-like enzymes (data not shown).

Alfalfa weevil gut extracts also were assayed with synthetic substrates conjugated to p-nitroanilide (Fig. 5). Proteinases in the gut extract hydrolyzed BAPNA, SAAPFpNA, and LpNA. At low substrate concentrations (<0.3 mg/ml), hydrolysis of BAPNA resulted in an increase of p-nitroaniline

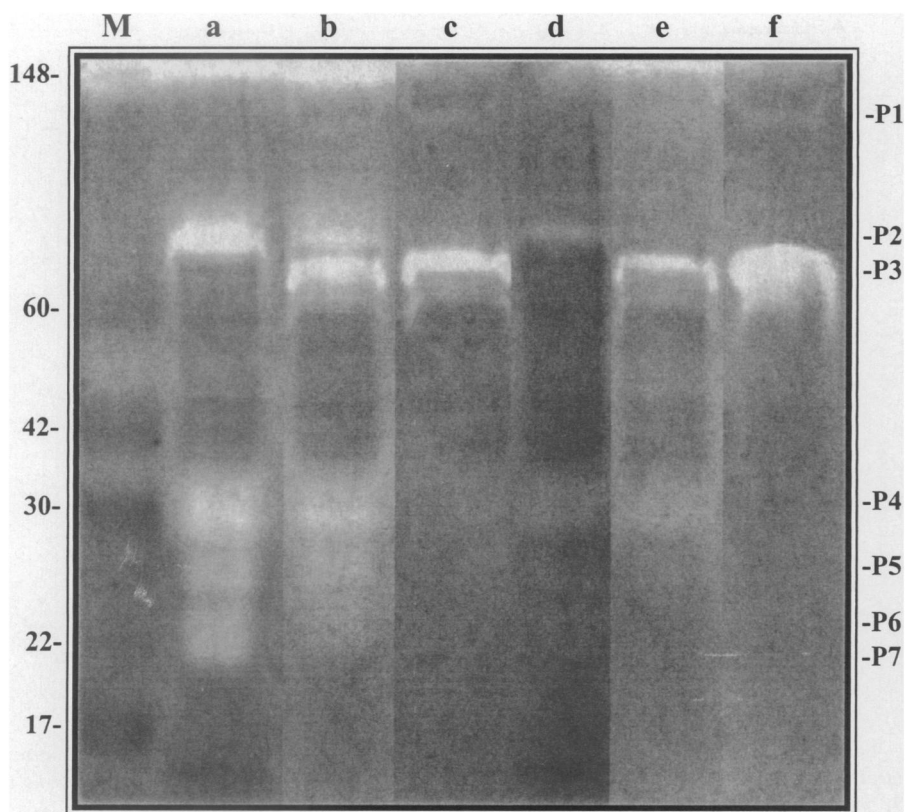


Figure 4. Zymogram analysis of alfalfa weevil gut extract at pH 4, 7, or 10, as indicated at top of gel, with or without L-cysteine, as indicated at bottom of gel. M: molecular mass markers. Proteinase activities (clear areas on the gel) were numbered P1 to P7, with higher number correlating to faster migrating proteins. Because of casein in gel, markers are included only as point of reference and cannot be used for absolute molecular mass determinations.

product, whereas at higher concentrations of substrate, the amount of BApNA hydrolysis decreased. Higher substrate concentrations resulted in a slight increase in LpNA hydrolysis. The greatest hydrolysis of substrate was achieved with SAAPFpNA, suggesting the presence of chymotrypsin-like proteinases. Although BApNA is a substrate for trypsin-like enzymes, it is possible that the activity observed is the result of other proteinases that recognize arginine at the P1 site, including some cysteine proteinases. Hydrolysis of LpNA is indicative of active aminopeptidases. Residual LpNA activity usually is associated with luminal enzymes but is more concentrated in the brush border membrane surrounding the lumen.

A significant chymotrypsin-like activity was observed using SAAPFpNA as a substrate in an activity blot analysis (Fig. 6). A strong activity was

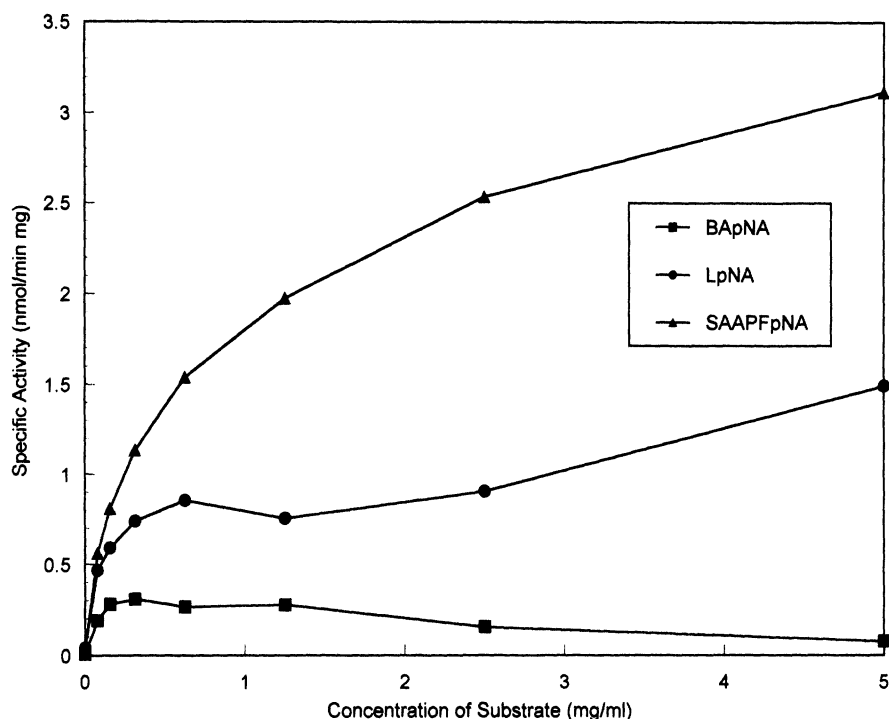


Figure 5. Specific activity (nmol/min per mg total gut protein) of alfalfa weevil gut proteinases using p-nitroanilide substrates BApNA, LpNA, and SAAPFpNA.

apparent between molecular mass markers of 105 and 208 kDa. A minor activity was observed at approximately 50 kDa. These activities may correspond to those identified as P1 and P3 in the zymogram analysis (Fig. 4). No activity was observed using the substrate BApNA. The activity of BApNA-hydrolyzing enzymes may have been below the threshold of detection.

Bioassays have shown that alfalfa leaves painted with Bowman-Birk trypsin-chymotrysin inhibitor or leupeptin resulted in significantly lower defoliation and survival of alfalfa weevils (Elden, 1995, 2000). Leupeptin-treated alfalfa reduced alfalfa weevil growth and survival for nine successive generations (Elden, 2000). Compensation by weevil larvae for chronic exposure to leupeptin was not observed. Based on the results of this study, we would argue that the efficacy of leupeptin is the result of the inhibition of both serine and cysteine proteinases. Furthermore, the inability of weevils to compensate for leupeptin toxicity may be a result of targeting two different proteinase subclasses. Combinations of serine and cysteine proteinase inhibitors also have been shown to be synergistic in reducing the growth and

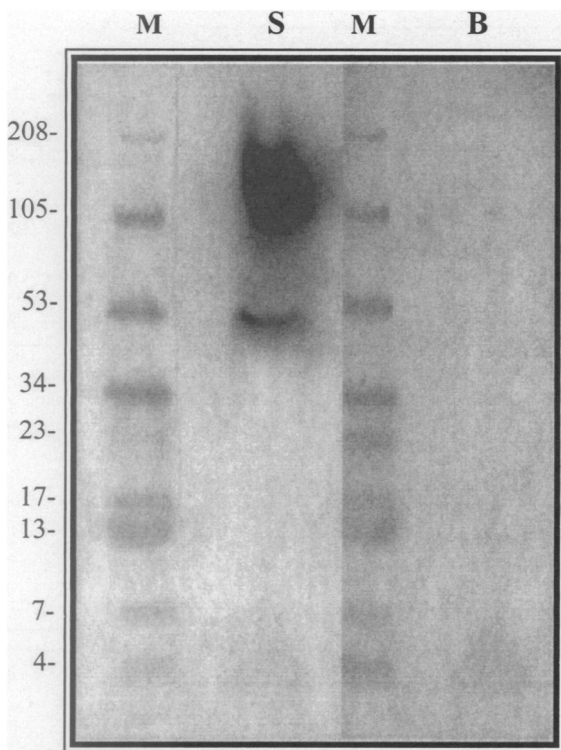


Figure 6. Activity blot analysis of alfalfa weevil gut extract, using substrates SAAPFpNA (S) or BApNA (B). M: molecular mass markers.

development of the red flour beetle, *Tribolium castaneum* (Herbst) (Oppert and others, 1993).

Examination of the physiological conditions of the alfalfa weevil gut indicated that serine and cysteine proteinases were important in weevil digestion. The gut pH of alfalfa weevil larvae was reported as 6.6 (Elden, 1995). Analyses of gut proteolytic activity at pH 7, closer to the physiological conditions in the gut, indicated that both serine and cysteine proteinase activities were significant.

Our evidence for both serine and cysteine proteinase activities in alfalfa weevil larvae indicates that a complex pattern of proteinases are involved in alfalfa weevil digestion. A complex digestive proteinase system was described for the sugar beet pest, *Aubeonymus mariaefrancisciae* Roudier, with activities similar to trypsin, chymotrypsin, elastase, cathepsin D, leucine aminopeptidase, and carboxypeptidases A and B (Ortego and others, 1998). In feeding assays with this pest, the most significant mortalities were achieved with diets containing combinations of two or three inhibitors. The

present study suggests that the digestive enzyme profile of alfalfa weevil larvae is similarly complex. Therefore, combinations of inhibitors targeting multiple proteinase classes will likely be more efficacious in promoting antibiosis in host plants. Inhibitors with strong chymotrypsin-inhibiting activity and cysteine proteinase-inhibitory activity may be good candidates for control of alfalfa weevil pests. Bioassays of weevil larvae with candidate inhibitors and inhibitor combinations will further substantiate their utilization in alfalfa transformation.

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